The broader health effects of Hepatitis B vaccination at birth:

An investigator-initiated randomized controlled trial in Guinea-Bissau

HBV0-NSE (Hepatitis B vaccination and non-specific effects) Trial Protocol

Version 1.2. September 17, 2025.

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STATEMENT OF COMPLIANCE

This document is a protocol for a randomized clinical research study. The study will be conducted in compliance with all stipulations of this protocol and the conditions of ethics committee approval (pending) from the Guinea-Bissau Committee of Ethics.

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PROTOCOL SYNOPSIS

Title: *The broader health effects associated with newborn Hepatitis B vaccination: An investigator-initiated randomized controlled trial in Guinea-Bissau*

Objective: To determine whether a single dose of monovalent Hepatitis B (HBV0) provided at birth or up to age 1 week (<8 days) is associated with severe morbidity in the first 6 weeks of life, and with atopic dermatitis (AD) by 2 years of age and neurodevelopment by 5 years of age, when compared with no vaccine (control). Both randomization groups will receive the recommended neonatal BCG and OPV vaccines, co-administered with HBV0 in the intervention group.

GLOSSARY OF ABBREVIATIONS

ABBREVIATION TERM

BCG, Bacille Calmette-Guérin

BHP, Bandim Health Project

CI, Confidence interval

Co-PI, Co-Principal Investigator

DSMB, Data and Safety Monitoring Board

HepB, Hepatitis B vaccine in infancy (part of the pentavalent vaccine)

HBV0, Hepatitis B vaccine, at birth dose

HDSS, Health and Demographic Surveillance System

HNSM, Hospital Nacional Simão Mendes

HR, Hazard Ratio

NSEs, non-specific effects (of vaccines)

PI, Principal Investigator

PS, Procurement Services (UNICEF)

OPV0, Oral Polio Vaccine, at birth dose

RCT, Randomized Controlled Trial

SDU, University of Southern Denmark

1. INVESTIGATORS AND FACILITIES

1.1 Trial Locations

Bandim Health Project, Bandim, Bissau, Guinea-Bissau.

Bandim Health Center, Bandim, Bissau, Guinea-Bissau.

Cumura Hospital, Cumura, Guinea-Bissau

Hospital Nacional Simão Mendes, Bissau, Guinea-Bissau.

Hospital Militar, Bissau, Guinea-Bissau

Bairro Militar Health Center, Bairro Militar, Bissau, Guinea-Bissau.

Plack II Health Center, Plack II, Bissau, Guinea-Bissau.

Hospital Casa Emanuel, Hafia, Bissau, Guinea-Bissau.

1.2 Study Management

1.2.1 Principal Investigator

The Principal Investigator (PI) for this trial is Dr Frederik Schaltz-Buchholzer, Assistant Professor, Bandim Health Project, University of Southern Denmark.

Co-PI is Professor Christine Stabell Benn, Bandim Health Project, University of Southern Denmark.

1.2.2 Statistician

The senior trial statistician will be Sebastian Nielsen, PhD, University of Southern Denmark.

1.2.3 Independent Data and Safety Monitoring Board

An independent Data and Safety Monitoring Committee (DSMB) will be established by the study group prior to the initiation of the trial. The DSMB's Terms of Reference will be established at the inception of the trial, outlining the Board's purpose, composition, responsibilities, and operational procedures. The DSMB will consist of a minimum of three senior clinicians and researchers, including at least one statistician and the DSMB will have authority to request modifications or termination of the trial if significant safety concerns or other significant arguments to the effect arise. Trial progress will be monitored through 6-monthly blinded progress reports submitted to the DSMB by the trial team. The DSMB will review any major proposed modifications to the trial (such as termination, or an increase in sample size) and provide advice to the PI and team. The DSMB will convene face-to-face or by conference call before the start of the trial, after half the proposed number of newborns have been enrolled and followed-up, and again after three-quarters have been enrolled – or as determined by the DSMB. The DSMB will review the trial design, informed consent process, data collection forms, safety measures, the statistical analysis plan, and visit the trial site once during the trial.

1.2.4 Trial Consortium

Assistant Professor **Frederik Schaltz-Buchholzer**, dept. of Clinical Research, SDU has 13 years of experience with the conduct of trials at the Maternity Ward[1–6] of Hospital Nacional Simão Mendes in Bissau, Guinea-Bissau. The Co-PI, Professor **Christine Stabell Benn**, dept. of Clinical Research, SDU is a co-discoverer of non-specific effects (NSEs) of vaccines and has three decades of experience with the conduct of RCTs in Guinea-Bissau, with established expertise in the bridging of vaccine epidemiology and immunology. The PI will lead the planning, conduct and reporting of the trial, in close collaboration with the Co-PI, who was the PIs PhD supervisor. The two have had a strong long-standing research collaboration since 2012.

Assistant Professor **Sebastian Nielsen**, dept. of Clinical Research, SDU, equally has long-term experience from the setting, including the conduct and reporting of RCTs[7–9], and will be the senior trial statistician.

MD, PhD **Cesário Martins**, National Institute of Public Health (INASA), Guinea-Bissau, has worked with the Bandim Health Project (BHP) for several decades and has extensive experience from the setting.

MD, PhD Fellow **Isaquel Silva**, dept. of Clinical Research, SDU, has worked with the BHP for several years and has experience from the conduct of clinical trials in the setting.

Professor **Peter Aaby**, Bandim Health Project, established BHP 47 years ago, discovered NSEs of vaccines and has invaluable experience from conducting RCTs in the setting.

Professor **Mihai Netea**, dept. of Internal Medicine, Radboud UMC, The Netherlands is a leading international expert in trained innate immunology[10,11] that has furthermore conducted several RCTs of vaccine NSEs and spearheaded key immunological discoveries within NSEs and trained immunity.

1.3 Sponsor

The Sponsor of this trial is the University of Southern Denmark.

1.4 Funding and Resources

External funding to finance the trial is necessary to fund the activities and has been applied for. External funders will have no role in the planning, conduct, or reporting of the trial.

2. INTRODUCTION AND BACKGROUND

2.1 Background Information

WHO recommends three vaccines at birth in low-income settings: Bacille Calmette Guérin (BCG) vaccine against tuberculosis, oral polio vaccine (OPV0) and hepatitis B vaccine. While much is known about the overall health benefits of BCG and OPV at birth, less is known about hepatitis B vaccine. The present study therefore focusses on investigating the overall health effects of hepatitis B vaccine at birth (HBV0).

Non-specific effects of vaccines. At the Bandim Health Project (BHP), a long-standing research collaboration between Guinea-Bissau's Ministry of Health and the University of Southern Denmark (SDU), 47 years of population data has uncovered that vaccines can have ***non-specific effects*** **(*NSEs***, also at times referred to as *off-target, heterologous, pathogen-agnostic or secondary vaccine effects*), i.e. beneficial or detrimental effects not explained by the specific protection against the targeted disease.[12] NSEs are at least partially mediated by induction of *trained immunity* resulting in a modified innate immune response to subsequent unrelated infections.[10,12] A meta-analysis of three randomized trials in Guinea-Bissau reported that administration of BCG-Denmark given soon after birth reduced neonatal mortality in low-birth-weight newborns by 38% (95% CI 17% to 54%) with infections and most notably neonatal sepsis accounting for the excess deaths among controls.[1,2] In addition, there were fewer hospital admissions with sepsis by 6 weeks of age among neonates randomized to BCG at birth. The beneficial effect of BCG-Denmark lasted until a different vaccine was given at 6 weeks of age. The first dose of OPV0 has been shown to further reduce mortality when given with BCG during the first two days of life, the mortality reduction comparing BCG+OPV vs BCG alone being 42% (10-62%).[13] This effect was especially evident for males and OPV was especially efficacious against fatal respiratory infections.[14] These findings led to national vaccine policy changes in Guinea-Bissau and inspired related research globally.[4,15–19] In a recent cluster-RCT from Guinea-Bissau, providing BCG + OPV0 at home visits within 72 hours of birth was associated with a 59% (8% to 82%) reduction in early infant mortality, when compared with home visits without provision of vaccines.[20]

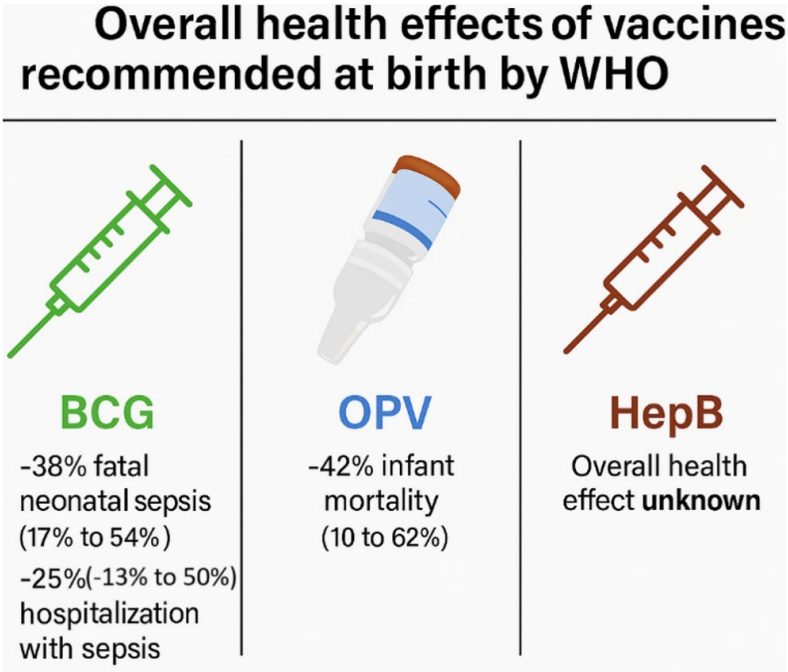
Hepatitis B. Hepatitis B virus is a major global health concern, not least in sub-Saharan Africa, where it is a leading cause of chronic hepatitis, cirrhosis and fatal liver cancer.[21–23] Infection acquired perinatally from mother to child (vertical transmission) or during early childhood (horizontal transmission) carries an elevated risk of chronic infection and severe disease downstream.[21]

Hepatitis B vaccination. Pentavalent vaccines provided at 6, 10, and 14 weeks of age provide protection against horizontal hepatitis B transmission[24]. Hepatitis B vaccination at birth (**HBV0**) is the most effective approach to prevent vertical transmission.[21]

A systematic Cochrane review of randomized controlled trials (RCTs) has shown that HBV0 reduces vertical transmission by 72% (60%-80%), but noted limited data on adverse events.[24] The use of HBV0 is especially important in countries with a high prevalence of hepatitis B surface antigen (**HBsAg**), yet WHO recommends universal HBV0 (including in neonates with a birth weight <2,000g and premature neonates), also in high-income countries.[21] Only 15 African countries have implemented HBV0 due to logistical and contextual barriers and the lack of benefit when the mother is HbsAg-negative.[24,25] Most high-income countries only provide targeted HBV0 to babies born to HBsAg-positive mothers.[26] The Health Ministry in Guinea-Bissau is planning to add HBV0 to the newborn vaccination program of Guinea-Bissau in 2027, in addition to the currently recommended BCG and OPV. Notably, since most African countries including Guinea-Bissau have no hepatitis B screening program, HBV0 is given to all neonates, even though it is only needed by those whose mothers are hepatitis B carriers. The majority of children are well protected against horizontal transmission by the three HepB vaccines included in the pentavalent vaccines at 6, 10 and 14 weeks of age. This makes it particularly important to ensure that there are no negative effects of HBV0.

HBV0 and non-specific effects
Hepatitis B vaccine was developed in 1982[21] and it has never been investigated whether HBV0 is associated with NSEs – beneficial or detrimental (**Figure 1**).

Figure 1. Effects on overall health of at-birth vaccines recommended by WHO.


Overall health effects of vaccines recommended at birth by WHO
BCG
-38% fatal neonatal sepsis (17% to 54%)
-25%(-13% to 50%) hospitalization with sepsis
OPV
-42% infant mortality (10 to 62%)
HepB
Overall health effect unknown

This is a critical evidence gap for an effective and universally recommended intervention, particularly since two observational studies from West Africa have suggested that HepB vaccines provided later in infancy may be associated with negative NSEs, and in line with what has been seen for other non-live vaccines, this was particularly among females.[27,28]

HBV0 and atopic dermatitis

Several studies have found that delaying the first dose of aluminum-adjuvanted pentavalent vaccine is associated with a reduced risk of developing AD. In Australia, diphtheria-tetanus-pertussis (DTP) aluminum-adjuvanted vaccine is recommended at 2 months of age. In a study of 4,433 Australian children, receiving DTP with one month's delay was associated with reduced risk of eczema (aOR: 0.57; 95% CI: 0.34-0.97, P = 0.04) and reduced use of eczema medication (aOR: 0.45; 95% CI: 0.24-0.83, P = 0.01).[37] In a subsequent study of 143,429 Danish children, for whom DTP is recommended at 3 months of age, one month's delay was also associated with a reduced risk of eczema (aRR 0.94; 95% CI, 0.91-0.97), which was further reduced if the second dose was delayed as well, aRR=0.88 (95% CI, 0.82-0.93).[38]

In the Danish study mentioned above[36], it can be deduced that the incidence rate of AD among children who did not receive aluminum-containing vaccines was 0.6% (229/36,678), vs 0.9% (22,749/2,666,948) for those that had received an aluminum-containing vaccine. While not adjusted for confounders, this provides a crude relative risk of 0.73 (0.64-0.83) for those who did not get aluminum-containing vaccines vs. those who received aluminum-containing vaccines. Similar analyses point in the same direction for the combined group of atopic and allergic outcomes, the relative risk being 0.81 (0.75-0.89).

HBV0 and neurodevelopment

Concerns have been raised regarding providing an aluminum containing vaccine within 24 hours after birth.[29] Aluminum is known to be a neurotoxin.[30–32] This is particularly relevant in infancy[33] and aluminum adjuvants have been speculated to be associated with neurodevelopmental disorders.[34,35] A recent Danish study[36] of 1,224,176 children found no association between the cumulative dose of aluminum received by age 2 years and subsequent development of neurodevelopmental disorders, but Denmark does not use at-birth vaccines; the first dose of aluminum-containing pentavalent vaccine is provided at 3 months of age, so the study does not exclude any association between at-birth aluminum exposure and the risk of downstream neurodevelopmental disorders. This research question has to our knowledge never been addressed.

Current plans for HBV0 in Guinea-Bissau

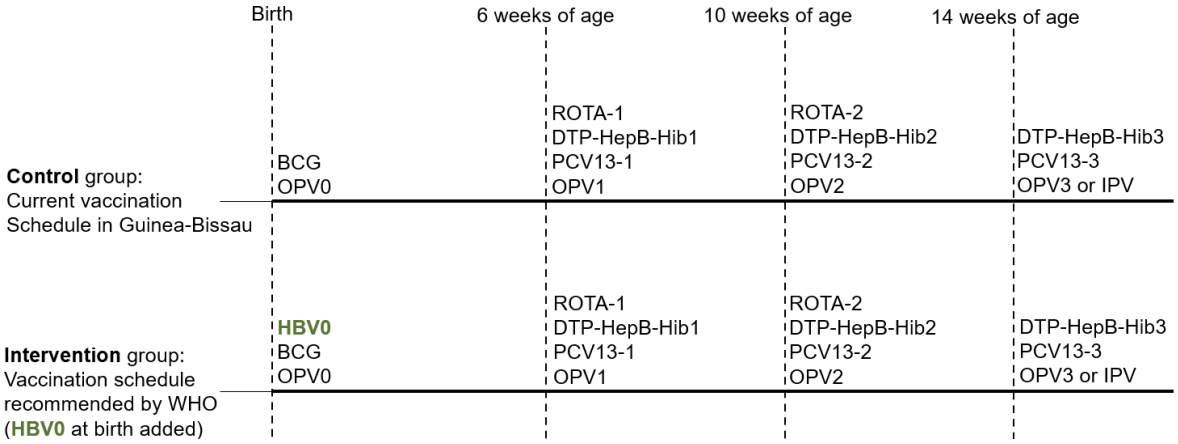
In a small cross-sectional study utilizing blood samples from children aged 18 months, who were born before HBV vaccination was introduced in Guinea-Bissau in 2008, 11% (21/187) were infected with Hepatitis B (HBsAg-positive) and 5% had recovered from infection.[39] A recent population-based study conducted at BHP documented a high (18.7%) prevalence of HBsAg in urban Bissau[40], making vertical transmission a relevant public health concern which could be addressed by HBV0 vaccination. The Ministry of Health in Guinea-Bissau plans to introduce HBV0 for all newborns in 2027. There is therefore a **unique window of opportunity to conduct a large-scale RCT evaluating the overall health effects of HBV0** before vaccination becomes national policy. The proposed trial would address the significant knowledge gap regarding the overall health effects of HBV0.

2.2 Rationale for Current Study

The available evidence suggests that BCG and likely also OPV, especially given in combination, reduces all-cause mortality in neonates in Guinea-Bissau, due to fewer cases of fatal sepsis and fewer respiratory infections.[1,2,13] Adding monovalent Hepatitis B vaccine to the at-birth schedule would address the relevant public health threat of vertical Hepatitis B virus transmission, but the effect on overall health is unknown.

Concerns have been raised in relation to potential effects on the risk of unrelated infections (NSEs), neurodevelopmental disorders as well as AD. A randomized trial that compares the current neonatal vaccination schedule in Guinea-Bissau, BCG + OPV0, with the WHO-recommended schedule of BCG + OPV0 + HBV0 (**Figure 2**) is needed to test whether healthy newborns given HBV0, co-administered with BCG + OPV0, have an elevated risk of these outcomes.

Figure 2. The current vaccination schedule in Guinea-Bissau up to 14 weeks of age (control, top) and the WHO-recommended vaccine schedule (intervention, bottom)



Abbreviations: BCG, Bacille Calmette-Gúerin; OPV, Oral Polio Vaccine; HBV0, Hepatitis B vaccine at birth; HepB, Hepatitis B vaccine in infancy; ROTA, Rotavirus vaccine; DTP, Diphtheria-Tetanus-Pertussis; Hib, Hemophilus Influenzae; PCV, Pneumococcus vaccine; IPV, Inactivated Polio Vaccine.

2.3 Research Questions

Does the administration of a single dose of neonatal monovalent HBV0 to newborns below 1 week of age reduce or increase all-cause mortality and non-fatal admission with infection (composite outcome) in the first 42 days of life?

Does the administration of a single dose of neonatal monovalent HBV0 to newborns below 1 week of age affect the risk of AD by 2 years of age?

Does the administration of a single dose of neonatal monovalent HBV0 to newborns below 1 week of age affect neurocognitive outcomes by 5 years of age?

Are there sex-differential non-specific effects of HBV0?

3. STUDY OBJECTIVES

3.1 Primary Objective

We will investigate whether the administration of a single vaccine dose of pediatric monovalent HBV0 provided within 1 week of birth is associated with the composite outcome of all-cause mortality and severe morbidity (non-fatal hospital admission with infection) within 42 days after birth, before other vaccines are administered.

3.2 Secondary Objectives

To investigate the effect of HBV0 on AD by 2 years of age.

To investigate the effect of HBV0 on neurodevelopment by 5 years of age.

For all outcomes, it will be assessed whether there are sex-differential effects of HBV0.

4. STUDY DESIGN

4.1 Type of Study

The study will be a single-blind, multi-center, phase IV randomized controlled trial enrolling healthy neonates that are ≤1 week of age. The main recruitment center will be the Maternity Ward of Hospital Nacional Simão Mendes, which has approximately 8,000 births/year. Pending funding acquisition and approval from the suggested recruitment sites, additional recruitment units will be Hospital Militar (~1,000 births per year), Hospital de Cumura (~700 births per year), Bandim Health Center (200 births per year). All inclusion sites are located in the greater Bissau area of Guinea-Bissau. We aim to recruit at least 14,500 children (see sample size calculation) during a 2-year period. Eligible newborns will be randomized 1:1 to receive 1 neonatal dose of monovalent HBV0 co-administered with BCG + OPV0 vs only BCG + OPV0.

Trial design and participant recruitment: Eligibility screening will occur at discharge from the birth facility, or at the facilities when a newborn born elsewhere comes for vaccination. In the unlikely case that the mother is a known Hepatitis B carrier, the child will be excluded and receive Hepatitis B vaccine. Newborns are eligible if they are clinically stable and unvaccinated. Moribund neonates and children with major malformations will be excluded. Informed written consent will be obtained from mothers/guardians following an oral explanation in Portuguese Creole and a written trial explanation in Portuguese. Participation is voluntary, and families may withdraw from the trial at any time. Baseline information such as maternal and neonate mid-upper-arm circumference, maternal BCG scar status, socio-economic factors and phone contacts for the mother, the father and family members will be collected, as done previously.[3,5]

Randomization and blinding: Randomization will be performed using sealed envelopes prepared in blocks of 24, stratified by sex and place of inclusion. After the informed consent procedure, the mother/guardian selects a sealed envelope allocating the neonate to either the intervention group (HBV0) or the control group (no vaccination). Due to logistical constraints, the inclusion team will not be blinded to group allocation. All outcome assessors involved in follow-up procedures will be blinded, ensuring objective outcome assessment while maintaining operational feasibility.

Intervention: All enrolled neonates will receive BCG (0.05 mL intradermal injection in the left deltoid) and OPV0 (2 oral drops), in accordance with national guidelines. The intervention group will additionally receive 0.5 mL of monovalent HBV0 intramuscularly in the anterolateral aspect of the thigh, with no placebo used in the control group. BCG and OPV are currently provided to all newborns at HNSM on weekdays, aside from situations with national vaccine stockout, which are rare. At the smaller hospitals and health centers, OPV is provided daily on weekdays while BCG is typically

available on one vaccination day during the week, to reduce vaccine wastage. The trial team will ensure that the necessary vaccines (BCG, OPV, HBV0) are available on all days of the week (including holidays and weekends) at HNSM and on normal workdays (excluding holidays and weekends) at the smaller inclusion sites.

4.2 Follow-up Measures

To ensure comprehensive outcome data, follow-up of all children will be conducted through 4 complementary mechanisms, including telephone interviews (**A**), home visits (**B**), hospital surveillance (**C**), AD by 2 years of age (**A, B, D**), and neurodevelopmental assessment by 5 years of age (**E**).

A) Telephone interviews at age 1 week, 6 weeks, 6 months, 1 year and 2 years of age.
Families will be contacted to assess vital status, hospital admissions, adherence to the recommended routine vaccination schedule (at 6, 10 and 14 weeks), and morbidity. If an infant has died, brief information will be collected regarding the circumstances such as date, location (home or hospital), and symptoms. In prior BHP trials, 99% of families provided at least 1 telephone contact, and >95% successfully provided data on vital status by 6 weeks of age (**Table 1**).[5]

Table 1. BCGSTRAIN II[5] Telephone follow-up data.

Mother supplied telephone number(s) at inclusion	99%
Follow-up success rate (combined for 6-week and 6-month calls)	95%
Successful 6-week follow-up if two phone numbers provided	88%
Successful 6-week follow-up with ≥three phone numbers provided	97%

This demonstrates the feasibility and minimal loss to follow-up with telephone-based interviews in our setting.

B) Cohort of BHP HDSS study area infants: home visit by 2 months and 12 months of age
Approximately 15% (~2,100) of the newborns will reside in BHP's Health and Demographic Surveillance System (HDSS) study area. These infants will receive a home visit at 2 and 12 months of age to assess survival status, BCG scar status and morbidity incidence, as done in previous trials.[1,3–5] In addition, the children will be asked about and inspected for signs of atopic dermatitis, and in case it is detected, the child will be referred to the AD specialist (see below). For HDSS infants that die, a verbal autopsy will be performed 3 months after the death.

C) Hospital admission surveillance
All admissions to the national hospital's pediatric ward HNSM are monitored and entered daily by a dedicated BHP team.[2,41–43] Parental names and telephone numbers are registered for all admissions and will be used for data linkage and data confirmation. Admission charts linked via a unique trial participant vaccination card sticker ID include the diagnosis and admission outcome. For approximately 60% of admitted infants, data from the vaccination card including the unique ID is documented during the admission. To identify remaining admissions not registered with the unique trial ID, a previously validated standardized probabilistic record matching protocol will be applied.[2,41] All admissions will be manually vetted by a blinded senior researcher. To assess the likely cause of death or admission, we will use both hospital data, telephone follow-up data, and verbal autopsy data from the BHP study area.

D) Assessment of AD
At the telephone contacts and HDSS home visits, we will ask the mother whether the child has an itchy rash. If the caregiver reports that the child has scratched repeatedly and/or experienced sleep disturbances due to itching and the family resides in the greater metropolitan area of Bissau, a home

visit will be offered by a physician experienced in diagnosing AD at a time point where the child suffers from the rash.

At the home visit, the physician will perform a clinical examination to assess whether the child has AD or another condition. Appropriate treatment will be initiated, and guidance will be given for clinical follow-up. At follow-up visits it will be ascertained if the treatment has helped, and/or if further/other treatment is needed. To ensure power to establish whether HBV0 has an effect on the accumulated incidence of AD, we will conduct home evaluation of AD cases until 200 AD cases have been diagnosed in the control group (see power calculation below). The data on 1) self-reported AD symptoms and 2) physician-diagnosed AD will be reported separately for the AD outcome.

E) Neurodevelopmental assessment
A subgroup of 500 BHP HDSS children will receive a neurodevelopmental assessment at 5 years of age, provided informed consent. The evaluation will take place at the local health center.

4.3 Primary and Secondary Outcome Measures

Primary outcome: Mortality and morbidity
The composite outcome of all-cause mortality and non-fatal admission with infection from randomization to 42 days of age (before the next routine vaccination) in the group given HBV0 compared with the control group.

Secondary outcomes: Neurodevelopmental disorders and AD
AD by 2 years of age
In a subgroup followed to 2 years of age (see sample size calculation): cumulative incidence of AD symptoms (self-reported at home visits or telephone follow-up) and cumulative incidence of AD diagnosed by an MD with special training.

Neurodevelopment by 5 years of age
In a subgroup of 500 infants followed to 5 years of age: cognitive outcomes assessed by the Mullen Scales of Early Learning and the Kaufman Assessment Battery for Children. These tests have been tested for validity in Sub-Saharan African children.[44–47]

The outcomes will be further explored to assess the effect by subcategory of outcome and by age (**Table 2**).

Table 2. Trial outcomes and data sources

Primary outcome	**Whom**	**Age assessed**	**Data source(s)**
Composite outcome: all-cause mortality and first admission due to non-fatal infection	All infants*	6 weeks	A, B, C
Secondary outcomes			
I: Self-reported AD and II: physician-diagnosed AD	I: All infants. II: To 200 cases among controls*	2 years	A, B, D
Neurodevelopmental outcomes	500 HDSS children*	5 years	E
Outcome subcategories and by age			
All-cause mortality excluding accidents and burns	All infants	6 weeks, 6 months	A, B, C
In-hospital case-fatality (all-cause, infectious diseases)	All infants	6 weeks, 6 months	C
Hospital admission at HNSM for major diseases groups (neonatal conditions aside from sepsis, respiratory infections, bronchitis, diarrheal diseases, malaria, malnutrition-related diseases)	All infants	6 weeks, 6 months	C

* See sample size calculation section below for further information.

4.4 Number of Subjects (Sample Size)

Based on a recently completed trial of BCG-Denmark+OPV0 vs BCG-Bulgaria+OPV0[6], conducted with the proposed setup and follow-up methodology, we anticipate a 6-week mortality rate of 1.4% and a non-fatal infectious disease admission rate of 2.6%, for a composite risk of 4% in the control group. The number of dropouts in previous trials has been low, and the composite risk of 4% is based on the collected data in the most recent trial[6], e.g., the rate accounts for loss to follow-up. If the rate of the composite outcome of all-cause mortality and non-fatal hospital admission with infection is 4% (0.04) in the control group, and HBV0 increases the rate of the composite outcome by 25% (to 1.25 x 0.04 = 0.05) or decreases the rate by 22% (to 0.78 x 0.04 = 0.0312), to have 80% power to detect such changes for the primary outcome, with alpha error <0.05, the required sample size is ≥14,368 newborns, 7,184 in each group, from sampsi .050 .040, a(0.05) p(0.80) and sampsi .040 .0312, a(0.05) p(0.80) in Stata/BE version 18.0. For simplicity, enrolling 14,500 newborns is expected to be possible before HBV0 is introduced in Guinea-Bissau. With a composite outcome prevalence of 4% (0.04), means that the number of deaths and non-fatal infectious disease admission expected is 7,250 x 0.04 = 290. With 290 events in the control group, expected to be reached when 7,250 newborns have been recruited in each trial arm, the trial has 80% power to detect if HBV0 has a beneficial non-specific effect, decreasing the rate of the primary outcome by ≥22% or a detrimental non-specific effect, increasing the rate by ≥24%. This design ensures statistical power and precision to detect also minor NSEs of HBV0, with no known disadvantages to participants. The exact timepoint that the trial will end is determined by the time when the Ministry of Health introduces HBV0 in Guinea-Bissau. We will therefore continue the trial until HBV0 is introduced in Guinea-Bissau or when 25,000 neonates have been enrolled in the trial, whichever comes first.

For AD, little is known about the prevalence in Sub-Saharan Africa, but the available data indicate prevalence rates similar to European Union countries, e.g., approx. 12% among 5-year old children.[48] With an anticipated cumulative incidence by 2 years of age of 10%, there would be 725 cases of AD in the control group if we recruit 7,250 children in each arm of the trial. We aim to conduct home visits to identify cases of AD until 200 cases have been diagnosed among controls, which would provide power to detect a 28% increase (to 256 intervention group cases) in the risk of AD for children who receive HBV0 and a 25% decrease (to 150 intervention group cases).
For cognitive outcomes, a sample size of 500 will make this study a large study with power to detect clinically meaningful differences in cognitive abilities between children who did or did not receive HBV0.[44]

4.5 Expected Trial Duration

We expect to be able to enroll 14,500 newborns within 1.5 years (**Table 3**). Follow-up will be until 2 years of age for mortality/morbidity. Follow-up for the main outcome will be finished ~2 years after trial initiation (~1½ years of enrolment and 6 months of follow-up). The first 500 children recruited from the HDSS will be followed to 5 years of age for cognitive outcomes. For this reason, the trial data collection should be finished within <7 years.

Table 3. Trial timeline

Period	Q4 2025	2026	2027	2028	2029	2030	2031
Events	Enrolment of 14,500 children						
Assessment for morbidity and mortality to 6 months	Ongoing	Ongoing	Ongoing	Assessment complete (n=14,500)			
Assessment for neurodevelopmental disorders by 5 years of age						Ongoing	Assessment complete (n=500)
Assessment for AD by 2 years of age	Ongoing	Ongoing	Ongoing	Ongoing	Ongoing	Assessment complete	

4.6 Planned seroprevalence substudies nested in the trial

To further shed light on the combined impact on overall health of providing HBV0, we plan to conduct 2 serosurveys in the cohort, pending funding acquisition and separate approval from the Guinea-Bissau ethics committee:

1. **Prevalence of HBsAg among women given birth in Bissau**. A previous study indicated a high (18.7%) prevalence of HBsAg.[39] Using a rapid diagnostic test (RDT) for HBsAg[53] with a drop of blood collected by finger prick, we will assess the prevalence of HBsAg. If the prevalence is ~19%, we would need to do 250 RDTs to measure the prevalence with a confidence interval of +-5%, e.g., a prevalence of 19% (14% to 24%). The HBsAg testing will be done per separate informed consent, before the child is enrolled in the main trial. For mothers that test positive, their neonate will be excluded from participation in the trial and offered HBV0, along with BCG and OPV, to protect the neonate from hepatitis B infection.

2. **Prevalence of HBsAg among children enrolled in the trial by 5 years of age.** At the 5 year follow-up procedure for cognitive testing, we will offer the family that their child participates in a serosurvey where we will measure the prevalence of HBsAg using an RDT.[53] We will sample 250 children and if the true prevalence of HBsAg is 13%, this will give us power to detect the actual prevalence with a precision of +-4%.

5 STUDY TREATMENTS

5.1 Treatment Arms

5.1.1 Description

Both groups will be vaccinated at discharge from the maternity ward or at the health center where they were recruited, or, in the case of neonates born at home, when they are brought to the health facility for their first vaccinations. All participants will receive BCG and OPV0 plus either HBV0 or no HBV0.

5.1.2 Dosage and Route of Administration

Intervention: All enrolled neonates will receive BCG (0.05 mL intradermal injection in the left deltoid) and OPV (2 oral drops) at hospital or health center discharge, in line with national guidelines. OPV and BCG will be provided through the national vaccination program to the HNSM inclusion center. For use at the other inclusion centers, and in case of shortage of vaccines, the BHP will procure OPV and BCG for the trial via UNICEF's Procurement Services (PS), ensuring that OPV and BCG is available for daily vaccination at all trial inclusion sites. BHP has a Memorandum of Understanding with UNICEF (partner code: N12038) and have previously procured vaccines and medical equipment via UNICEF PS with delivery to Guinea-Bissau. The intervention group will additionally receive a 10 mcg dose (in 0.5 ml suspension) of monovalent HBV0 (HepatitisB pediatric subunit vaccine, Serum Institute of India (SII))[49] intramuscularly in the anterolateral aspect of the thigh. In case the SII HBV0 vaccine cannot be acquired, Euvax B (LG Chem Ltd)[50] or BEVAC® (Biological E. Limited)[51] will be sourced. All 3 HBV0 vaccine preparations are prequalified by WHO.

6 SUBJECT ENROLLMENT AND RANDOMIZATION

6.1 Recruitment

All unvaccinated and healthy neonates will be eligible for the trial provided that written informed consent is obtained and they fulfil none of the exclusion criteria.

Eligibility screening will occur at the recruitment centers, either at discharge from the Maternity ward or when neonates are brought there for their first neonatal vaccines. Neonates born at home, presenting at a recruitment center, are thus eligible. Informed written consent will be obtained from mothers/guardians following an oral explanation of the trial in Portuguese Creole and a written explanation in Portuguese. Participation is voluntary, and families may withdraw from the trial at any time. If the family declines to participate or the child is not eligible for any other reason, the inclusion team will offer to vaccinate the child with BCG and OPV, unless there are known contraindications for vaccination. Baseline information such as maternal and neonate mid-upper-arm circumference, maternal BCG scar, socio-economic factors and phone contacts for the mother, the father and family members will be collected, as done previously.[3,5]

6.2 Eligibility Criteria

6.2.1 Inclusion Criteria

Neonates can be enrolled if they are present at one of the recruitment centers, are clinically stable and the neonate's family (mother, father or guardian) has provided informed consent for participation.

6.2.2 Exclusion Criteria

Participants fulfilling any of the following will be excluded:

- Age more than 1 week ($\geq$8 days of age).
- Already received any vaccine.
- Is moribund as determined by the BHP inclusion supervisor or the staff at the inclusion center.
- Has a major visible malformation.
- The mother is a known hepatitis B carrier, in which case the neonate will be immediately vaccinated with BCG, OPV0 and HBV0 by our team.

6.3 Randomization Procedures

After informed consent has been obtained, enrolment data will be documented on a written Case Report Form (see below). From a collection of sealed randomization envelopes, prepared in blocks of 24 and stratified by sex and place of inclusion, the mother selects a sealed envelope allocating the neonate 1:1 to the intervention group (HBV0), or the control group (no 3rd vaccine). To avoid confusion at subsequent follow-up, twins of the same sex are only randomized once and allocated the same treatment (the mother only draws one sealed randomization envelope), whereas twins of different sex each will be randomized according to their sex. In the case that triplets of different sex are enrolled, the two triplets with the same sex will be randomized once (together), and the one triplet with a different sex will be randomized alone.

6.4 Blinding Arrangements

Due to logistical constraints, the inclusion teams at each inclusion center will not be blinded to the randomization allocation. Since there is no placebo vaccine, the mother/guardian will also not be blinded to the randomization allocation. However, all outcome assessors involved in follow-up procedures will be blinded, ensuring objective outcome assessment while maintaining operational feasibility in the setting. The senior trial statistician, who will conduct the final analysis of the trial data, will be blinded to randomization allocation until the DSMB decides to unblind the trial.

6.5 Breaking of the Study Blinding

The trial will be blinded until study end, or until the DSMB decides to unblind the trial.

6.6 Subject Withdrawal

Because HBV0 is given in a single dose, the parents will not be able to withdraw their child from treatment except if within the short time interval between randomization and vaccination. Consent can be withdrawn for follow-up procedures. Participants whose parents withdraw consent for their child's data to be used will not count towards the total number of participants, and they will not receive further follow-up calls or home visits.

6.7 Protocol Violations

Protocol violations will be recorded throughout the trial for participants in both treatment arms; they will be recorded in a trial log along with all other major events (e.g., health care system strikes, milestones, vaccine campaigns, and any other important observations).

6.8 Trial Closure

In principle, the trial is powered to be completed when 7,250 eligible newborns have been enrolled in each arm of the trial. In practice, we will continue enrollments until the Health Ministry of Guinea-Bissau introduces universal HBV0 or when 12,500 eligible newborns have been enrolled in each arm of the trial, whichever comes first.

7 ADVERSE EVENTS

BCG can cause axillary lymphadenitis, and very rarely this can ulcerate. Very rarely, in an infant with severe immunodeficiency, BCG can cause disseminated infection (*BCGitis*). OPV can cause vaccine-associated paralytic poliomyelitis diarrhea, fatigue and muscle weakness. For HBV0, the most common reactions are mild soreness, erythema, induration, fatigue, fever, malaise, influenza-like symptoms. Less common systemic reactions include nausea, vomiting, diarrhea, abdominal pain, abnormal liver function tests, arthralgia, myalgia, rash, pruritus, urticaria.[52] All the listed adverse events are rare or very rare. All 3 vaccines can cause allergic reactions in very rare circumstances. Each inclusion team will have adrenalin available along with guidelines for medical therapy to handle an acute anaphylactic reaction. All adverse events are captured through the outcome data collection.

8 STATISTICAL METHODS

8.1 Population to be analyzed

Data analysis will be conducted as intention-to-treat including all participants as randomized, apart from participants whose parents have withdrawn consent for their child's data to be used as part of this study, who will be excluded from analysis.

8.2 Statistical Analysis

The Statistical Analysis Plan (SAP) will be drafted by the trial group and submitted to the DSMB for approval. Upon approval and before initiation of data analysis, the SAP will be uploaded to an online repository. When requested by the DSMB, the trial data can be unblinded and analyzed according to the SAP.

The primary endpoint for the trial will be the composite outcome of all-cause mortality and first non-fatal infectious disease admission up to 42 days of age with censoring at the time of an event, loss to follow-up, or by 42 days of age, whichever comes first. Cumulative curves for mortality and hospitalization risk will be computed using the mean cumulative function, overall, and by sex. Person-years of risk will be calculated from enrollment (day, hour and minute of randomization). The risk of the single-event composite outcome (all-cause mortality or first non-fatal admission with infection) will be estimated using Cox proportional hazards models with age as the underlying time

scale, thus inherently adjusted for age and with stratification by sex, allowing for separate baseline hazards function by sex. The Cox models will provide hazard ratios (HRs) from single-event analyses of the composite outcome (all-cause mortality or first non-fatal admission with infection) up to 42 days of age (primary endpoint). Incidence rate ratios for secondary endpoints relating to morbidity (hospital admission at HNSM for major diseases groups) will be estimated using recurrent-event Andersen-Gill Cox proportional hazards models with robust standard errors and age as the underlying time scale. Infants will not be considered at risk of admission while admitted. Randomization will be stratified by sex and place of enrollment in blocks of 24; we will therefore include different underlying baseline hazard functions by sex and place of enrollment in the Cox models. The Cox regression models will furthermore account for the survival interdependency among twin pairs by using the Stata cluster command.

Tests for proportionality of hazard rates will be computed using Schoenfeld residuals, and by visual inspection of cumulative risk curves drawn using the Kaplan-Meier method.

In-hospital case-fatality risk will be estimated using binomial regression providing Risk Ratios (RRs), also using the Stata cluster command. In case there are fewer than five events in one group, Fisher's Exact Test will be used to compute a p-value.

Cognitive scores will be standardized and tested using linear regression models.

The cumulative incidence of atopic dermatitis will be investigated using binomial regression providing Risk Ratios (RRs), also using the Stata cluster command.

All analyses will be done overall and by sex using Stata software (StataCorp, College Station, Texas), with all estimates reported with 95% Confidence Intervals.

8.3 Subgroup Analyses

Within each of the 3 outcome categories, there will be assessment of outcomes by other ages and of outcomes assessed in more specific ways, as also outlined above.

Primary outcome: Mortality and morbidity

- The composite outcome of all-cause mortality and non-fatal admission with infection by 42 days of age and by 6 months of age
- All-cause mortality (excluding accidents and burns) by 42 days of age and 6 months of age.
- In-hospital case-fatality for all-cause and infectious disease admissions by 42 days of age.
- Morbidity: hospital admissions and in-hospital case-fatality rate at HNSM for major diseases groups (sepsis, respiratory infections, bronchitis, diarrheal diseases, malaria, malnutrition-related diseases) by 42 days of age and by 6 months of age.

Potential effect modification
Cox regression will be used to determine whether the effect of HBV0 is modified by the following potential effect modifiers (in addition to sex): vaccination campaigns, the BCG scar status of the mother at enrollment, inclusion weight ($\geq$2,500g, between $\geq$2,000g to <2,500g, and <2,000g), season, mode of delivery (vaginal or Caesarean section) and whether the baby was born at a hospital or elsewhere. We will conduct a supplementary analysis to assess HBV0's non-specific effects by age at enrolment (within 24 hours and $\geq$24 hours after birth), our hypothesis being that HBV0 will have the most marked effects among the youngest neonates. Supplementary analyses

will investigate whether there are seasonal effects, whether censoring for other health interventions such as vaccination campaigns modulates the effect, and whether there is an interaction with the maternal BCG scar status. For the composite outcome by 42 days of age and by 6 months of age, we will calculate the female/male ratio.

9 DATA MANAGEMENT

9.1 Data Collection

Data collection will be undertaken by the researchers and entered into databases designed for the trial by data entry clerks at the Bandim Health Project.

9.2 Data Storage

Data will be stored on site-specific departmental password-protected computer files and analyzed in de-identified but re-identifiable form.

9.3 Study Record Retention

All study data will be kept on password protected computer files for a period of 20 years.

10 ADMINISTRATIVE ASPECTS

10.1 Confidentiality

All study data will be kept on departmental password protected computer files for 20 years.

10.2 Modifications of the trial protocol

Unforeseen modifications of the trial protocol will be communicated to the DSMB and sent for ethics approval prior to implementation.

10.3 Participant Reimbursement

There will be no reimbursement for the participants.

10.4 Financial Disclosure and Conflicts of Interest

None of the investigators have a financial disclosure or conflict of interest.

11 USE OF DATA AND PUBLICATIONS POLICY

The trial results will be published in peer-reviewed journals. A summary in Portuguese and a copy of the trial report will be sent to the library of the National Institute of Health in Guinea-Bissau (INASA) and the results will be communicated to the National Committee of Ethics in Guinea-Bissau.

12 ETHICAL CONSIDERATIONS

The trial will be conducted in adherence to the ethical standards outlined in the Declaration of Helsinki. The ethical committee of Guinea-Bissau will review the trial design, informed consent process, data collection forms and safety measures.

Information about the trial will be provided in clear, understandable language considering the cultural and educational background of the mother/guardian, and the difficulties surrounding childbirth in Guinea-Bissau. It will be ascertained that parents or guardians fully understand the implications of trial participation.

It will be explained that all neonates (regardless of participation) are entitled to free consultations at the 3 health centers located within the HDSS of the Bandim Health Project, in Bandim, Belém and Cuntum. Children presenting for consultation will be given access to essential medications free of charge. BCG and OPV vaccination will be offered to newborns that are ineligible to participate or where the mother/guardian declined participation. Participants in the trial will be covered via the collateral insurance agreement of the University of Southern Denmark.

For the subgroup of children participating in the neurodevelopmental assessment, it will be emphasized that the results can help identify a child's strengths and areas where they might need additional support. Testing can help identify potential learning difficulties early on, allowing for timely interventions and support. It will be explained that cognitive tests are not the sole measure of a child's intelligence or potential. They provide a snapshot of specific cognitive skills at a particular time. Parents will be reassured that there is no need for children to prepare for the tests, and that the aim is to understand their current abilities, not to catch them out. It will be made clear that results are kept strictly confidential.

For the subgroup of children participating in the assessment of atopic dermatitis (due to an itchy rash), they will have access to a study MD with specialized training in diagnosing and treating atopic dermatitis.

The trial will examine the non-specific effects of HBV0 - a widely administered neonatal vaccine that is universally recommended by the WHO, also to neonates weighing below 2,000 grams and to premature neonates. We will use 1 of the 3 HBV0 vaccines that has been evaluated and approved by WHO (pre-qualification) to meet international standards of quality, safety and efficacy.

Half of the participants will receive HBV0 which they would otherwise not have received. HNSM is currently the only Maternity Center in urban Bissau where BCG and OPV are routinely administered daily, although not on weekends and public holidays. As a consequence of the trial, more children will have timely vaccination opportunities, as BCG and OPV vaccines will be provided within 7 days after birth at all recruitment centers on each weekday. Vaccination of these newborns would otherwise often be delayed and procuring vaccination can be associated with a financial burden for the household.[54,55] Given that RCTs indicate beneficial non-specific effects of both BCG[1] and OPV[13], all participants are thus expected to benefit from their participation in the trial. Whether the trial identifies that HBV0 is associated with positive, negative or no non-specific effects, it will be of high importance for vaccine policy, providing crucial input on how to best reduce child mortality and morbidity. Such data will be the best way to maintain vaccine confidence in the long run.

13 APPENDIX

13.1 Trial Inclusion Form.

Regno (birth number, HNSM): ____ Adhesive study number:____ Written study number:____

Date ___/___/20__ Inclusion assistant:_____ Place of enrollment:_____

The child already received a vaccine: Yes__ No__ Is sick or has a malformation: Yes__ No__

Age under 1 week: Yes__ No__ Can be included: Yes__ No__

Information about the parents

Relation to the child___(1: Mother, 2: Father, 3: Uncle/aunt, 4: grandparent, 5: Other)

The mother is alive: Yes___No___ If no, date of death: __/__/202__

Mother's name_____

Telephone number, Orange:_____ Telephone number, Airtel:_____

Does not have a telephone__ Does not remember telephone number__ House name_____ Age_____ Ethnicity_____ Schooling_____ From BHP area Yes__ No__ BHP address information: Neighborhood and zone (TaBz)_____ House number (CaMo)_____ Child ID (CNO)_____ Neighborhood (if not BHP area)_____

If from BHP area: the mother participated in the MATVAC trial: Yes__No__Unknown__ Mother's place of birth_____

Father's name_____

Telephone number, Orange:_____ Telephone number, Airtel:_____

Does not have a telephone__ Age_____ Ethnicity _____Went to school Yes___No___ Unknown__ Father lives with mother Y__ N__

Other contacts (relation to the child)

Name contact 1_____ Relation_____ Nº_____

Name contact 2_____ Relation_____ Nº_____

Name contact 3_____ Relation_____ Nº_____

Measurements

Mother: Arm circumference_____ mm Has BCG scar Yes__No__ Width___ Height___ Assistant__

Father: Arm circumference_____ mm Has BCG scar Yes__No__ Width___ Height___ Assistant__

Information about the child

Name_____

Birth date _____/_____/202_____ Time of birth ___:___

birthplace_____ C-section Yes__No__

Sex Male__ Female__ Twin Yes__No__ Twin number_____ Twin study number_____
Height____ Head circumference____ Admitted before enrolment Yes__No__ Where_____

If admitted, date of hospital admission ___/___/20__ Date of discharge ___/___/20__

Observations_____

Birth weight_____ Weight on day of inclusion_____

Confirmed the child's sex (assistant name):_____

The mother or guardian of the child provided consent for participation Yes__No__

Randomization

Date of randomization _____/_____/202___ Time of randomization ___:___

Randomization result: HBV0_____ Randomization result: No HBV0_____

Randomization lot number_____

Date of HBV0 vaccination: ___/___/ 202__ Time ___:___ Batch **HBV0**: _____ Expiry __/202__

Date of BCG vaccination: ____/____/ 202__ Batch **BCG**: _____ Expiry ___/202___

Date of OPV0 vaccination: ____/____/ 202__ Batch **OPV0**: _____ Expiry ___/202___

BCG vaccination papule: Height __ mm Width__ mm Vaccinator:_____wil__

BCG vaccine strain administered: ____ (Denmark: 1, Japan: 3, Bulgaria: 5)

Confirmed by: _____

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